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Induction of HPV16 Capsid Protein-Specific Human T C ll Responses by Virus-Like Particles

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It has been postulated that upon binding to a cell surface receptor, papilloma virus-like particles (VLPs) gain entry into the cytosol of infected cells and the capsid proteins L1 and L2 can be processed in the MHC class I presentation pathway. Vaccination of mice with human papilloma virus-like particles consisting of capsid proteins L1 and L2 induced a CD8-mediated and perforin dependent protective immune response against a tumor challenge with human papilloma virus transformed tumor cells, which express only minute amounts of L1 protein. Here we show that HPV16 capsid proteins stimulate a MHC class I restricted CTL response with human peripheral blood lymphocytes (PBL) *in vitro*. The vigorous response was specific for VLP-infected target cells and was MHC class I restricted. Moreover we show the presence of at least one HLA-A*0201 restricted CTL epitope within the HPV-16 capsid proteins by using a VLP-'infected' HLA-A*0201 transfected human cell line as target cells. These results demonstrated that VLPs can induce a HPV16 capsid protein-specific immune response in humans, allowing the monitoring of immune responses induced by vaccines based on chimeric VLPs carrying additional immunogenic peptides or proteins in therapeutical applications in human patients.

Key words: Cervical cancer / CTL / HLA-A*0201 / Human papilloma virus / Vaccine.

[a] Both authors contributed equally to this work.

Introduction

Cervical cancer in humans is strongly associated with infections with human papilloma virus type 16 (HPV-16; zur Hausen, 1991). Papilloma viruses attach to a cell surface receptor (possibly the α6β4 integrin; Evander *et al.*, 1997) and gain entry into the cell cytosol (Müller *et al.*, 1997). There the viral DNA is released and transported into the nucleus while the viral capsid proteins can presumably be processed and presented to the immune system via the MHC class I pathway. The observation that HPV capsid proteins L1 and L2 spontaneously form virus-like particles (VLPs) (Kirnbauer *et al.*, 1992, 1993) indistinguishable from normal viral capsids after production in insect cells opened the possibility for the use of VLPs as carriers for vaccines. The use of VLPs as a general carrier for immunogenic proteins, peptides or plasmid DNA have been reported and proven the versatility of the system as a vaccination tool (Greenstone *et al.*, 1998; Touze and Coursaget, 1998; Unckell *et al.*, 1997; Peng *et al.*, 1998). For example, after vaccination with chimeric VLPs containing the HPV-16 derived transforming protein E7, immunized mice were protected in a CD8 restricted and perforin dependent manner against subsequent tumor challenge with an HPV-16 E7 expressing tumor cell line (Greenstone *et al.*, 1998). Moreover, a similar protective immune response has also been induced by HPV-16 L1 VLPs. Mice vaccinated with VLPs consisting of L1 capsid proteins alone were protected against a subsequent tumor challenge with an HPV-16 induced tumor cell line expressing minute amounts of HPV-16 L1 proteins (De Bruijn *et al.*, 1998).

In this study, we tested whether the use of HPV-16 VLPs can also stimulate a HPV16 capsid protein-specific immune response in human PBL, since application of VLP based vaccines on human patients is already ongoing. After incubating non-adherent human HLA-A*0201 positive PBL with HPV16-L1 VLPs or HPV16-L1L2 VLPs *in vitro*, we have been able to show a VLP-specific immune response by T cell proliferation assays as well as T cell cytotoxicity assays with VLP-'infected' target cells. Moreover, by transfection of HLA-A*0201 negative human adherent RD cells with HLA-A*0201 and subsequently using these transfectants as targets for the effector cells, we show that at least a part of the cytolytic activity of the VLP-specific effector cells was HLA-A*0201 restricted. Our results demonstrate the strong immunostimulatory activity of HPV-16 VLPs, which are capable of stimulating an MHC restricted CTL response against the capsid proteins of HPV-16 *in vitro*.

Results

In order to test whether any HPV16 capsid protein-specific human T cell mediated immune responses (primary or secondary) could potentially be elicited *in vitro* we cultured human PBL in the presence of HPV16 VLPs. Three days after the start of the culture large clumps of proliferating cells could be observed in wells with HPV16 VLPs (Figure 1B), but not in control wells without any VLPs (Figure 1A).

The proliferation of PBL upon incubation with HPV16-L1L2 VLPs could also be demonstrated by ^{3}H-thymidine incorporation (Figure 2). Different numbers of non-adherent PBL were incubated in the presence or absence of HPV16-L1 VLPs or HPV16-L1L2 VLPs, and ^{3}H-thymidine incorporation was tested after 72 hours. As a control we used a preparation of mutated L1 protein, termed L1(152) capsomers, where the L1 amino acid sequence was changed at position 152 (Cys ⇒ Ser). These mutated L1 proteins still can form capsomers but are unable to assemble into intact VLPs as confirmed by electron microscopy (data not shown). As shown in Figure 2, cultures without any stimulus as well as cultures in the presence of mutated L1(152) capsomers did not show any significant ^{3}H-thymidine incorporation, meanwhile the cultures with HPV16-L1 VLPs or HPV16-L1L2 VLPs harbored large amounts of ^{3}H-thymidine indicating a pronounced proliferative response to the VLPs (results are shown for the male donor, but cells from the female donor gave identical results). Removal of CD8 cells by magnetic beads prior to incubation reduced the proliferative response by approximately 20% (data not shown), indicating at least a partial contribution of CD8 cells to the observed proliferation in the presence of HPV16 VLPs. The capsomers formed by mutated L1(152) proteins do not aggregate into intact VLPs and obviously are not able to induce proliferation of responder cells. The observed proliferative response *in vitro* in non-adherent human PBL against the HPV16 capsid proteins therefore is dependent on intact VLPs, either L1-VLPs or L1L2-VLPs.

We have previously shown that chimeric HPV16-L1L2 VLPs carrying the E7 protein can induce a CD8 mediated protective CTL immune response in mice. To test the hypothesis that the VLPs are able to induce a CTL response *in vitro*, which contributes partially to the observed prolif-

Fig. 1 Cell Clustering through incubation of PBL with HPV VLPs.
Non-adherent human PBL after 3 days of culture in the absence (A) or presence of HPV16 L1L2-VLPs (B) The experiment was repeated for two donors twice each with identical results; representative results are shown from one experiment. Human PBL were depleted from adherent cells and remaining non-adherent cells were incubated at 0.5 × 10^6 cells per well in 48-well plates containing 0.5 ml of medium with 5% human serum. Additionally 10 µg/ml of HPV16 L1L2-VLPs were added directly to the culture. Clumps of proliferating cells formed only in wells containing HPV16 L1L2-VLPs which were present through the whole culture period.

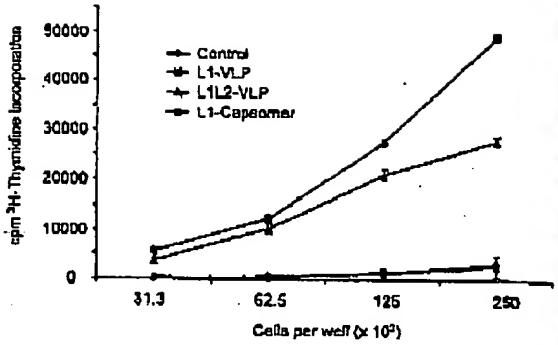

Fig. 2 HPV VLP Induced Proliferation of Human PBL.
^{3}H-thymidine incorporation of human PBL (non-adherent) after 72 hours culture in the absence or presence of HPV16 L1-VLPs, HPV16 L1L2-VLPs or mutated L1 protein [L1(152) capsomers], which do not form complete VLPs. The experiment was repeated for two donors twice with identical results; results are shown for male donor, results from the female donor were identical. Non-adherent human PBL were incubated in 96 well plates containing 0.2 ml of medium with 5% human serum and either 10 µg/ml of HPV16 L1-VLPs, HPV16 L1L2-VLPs, mutated L1(152) capsomers or no stimulus for 72 hours. Additional 25 µl medium were added containing 16 µCi/ml ^{3}H-thymidine and the cells were incubated at 37°C and harvested after 6 hours. Cells cultured in the presence of VLPs incorporated large amounts of ^{3}H-thymidine, while control cells without any stimulus or cells incubated in the presence of mutated L1(152) capsomers did not show any sign of proliferation.

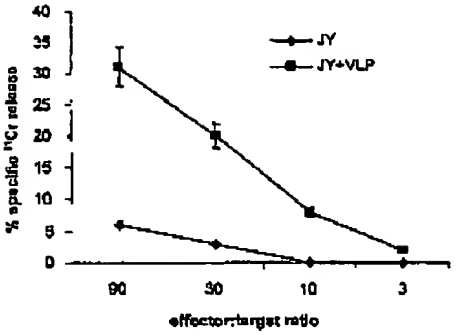

Fig. 3 HPV VLP Induced Human CTL.
Chromium release assay of cultured human PBL (results shown for female donor) in the absence or presence of HPV16 L1L2-VLPs against VLP-incubated JY target cells at different effector:target ratios. Non-adherent human PBL were cultured in medium containing 5% human AB serum. 10 µg/ml HPV16 L1L2-VLPs were added directly to the culture for 7 days. After restimulation with autologous non-adherent human PBL and HPV16 L1L2-VLPs, the remaining cells were tested against the [^{51}Cr]-labeled cell line JY loaded with HPV16 L1L2-VLPs at different effector:target ratios.

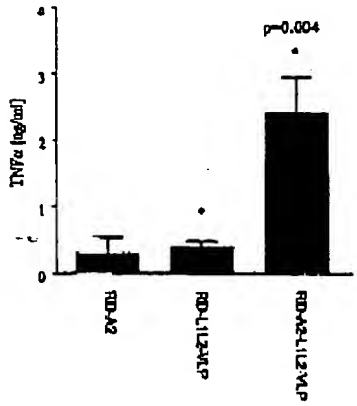

Fig. 4 HPV VLP Induced HLA-A*0201 Restricted Human T Cell Responses.
WEHI assay of effector cells recognizing VLP-loaded RD cells transfected with the human HLA-A*0201 gene. Adherent human RD cells were transfected with the cloned human HLA-A*0201 gene in pCDNA3 and cultured for 24 hours. Transfectants were incubated for 24 h in the presence of 10 µg/ml HPV16 L1L2-VLPs o.n. and subsequently incubated with effector cells (results shown for female donor). Supernatants were tested for the presence of tumor necrosis factor α (TNFα) in a standard WEHI assay. Only cells transfected with HLA-A*0201 and incubated with VLPs were able to activate the effector cells generated with VLP-incubated stimulator cells, while controls with either VLP-incubated cells or A*0201-transfectant cells alone did not induce the production and release of significant amounts of TNFα from the effector cells. The background value of RD cells alone (1.5 ng/ml) was subtracted from each value. The p-value was calculated from data (indicated by *) by two-sample Student's *t*-test assuming equal variances.

erative response, non-adherent human PBL were cultured in the presence of HPV16-L1L2 VLPs for one week. After re-stimulation for seven days we tested the remaining effector cells at different effector:target ratios in a chromium-release assay. We loaded human HLA-A*0201 positive lymphoma cells (JY) with HPV16-L1L2 VLPs overnight (o.n.) and used these cells in a cytotoxicity assay as targets for the cultured PBL. As shown in Figure 3, the effector cells from the *in vitro* culture specifically and efficiently lysed VLP-'infected' target cells, whereas non-infected target cells were hardly lysed, indicating the presence of at least one human CTL epitope within the HPV-16 capsid proteins.

The human PBL used in the experiment above are positive for the HLA-A*0201 allele and therefore we tested whether a part of the cytolytic activity of the effector cells was HLA-A*0201 restricted. Thus, we transfected HLA-A*0201 negative adherent human RD cells (Rhabdomyosarcoma cell line) with the human HLA-A*0201 gene and subsequently incubated the transfected cells with HPV16-L1L2 VLPs overnight. The RD cells were chosen because they can be transfected very efficiently (up to ~100% transfection rate, data not shown) and the transient expression of the introduced HLA-A*0201 gene is very high as detected with FACS staining (data not shown). The RD cells transfected with HLA-A*0201 and loaded with VLPs were able to activate the effector cells in the *in vitro* culture and induced a significant production and release of tumor necrosis factor α (TNFα) into the culture supernatant, as determined in a WEHI assay (Figure 4). The control experiment with VLP loaded RD cells or unloaded HLA-A*0201 expressing RD cells showed that these cells were not able to activate the effector cells and significant lower amounts of TNFα could be detected. Therefore we conclude that the *in vitro* cytolytic response is MHC class I restricted and that at least one HLA-A*0201 restricted CTL epitope is present in the L1 or L2 protein (the HLA-A*0201 is the only shared MHC class I molecule between effector and transfected RD cells).

Discussion

The results presented here clearly indicate the presence of CTL epitopes in the papilloma virus capsid proteins L1 and/or L2. Moreover human PBL are capable of mounting a MHC class I restricted immune response *in vitro* against capsid proteins upon presentation. These results extend the data on L1-specific CTL responses via HPV16 L1 recombinant vaccinia virus vectors in mice and protective CTL responses in mice against the L1 expressing tumor cell line C3, induced by immunization with HPV16-L1 VLPs (De Bruijn *et al.*, 1998; Zhou *et al.*, 1991). In addition, T cell responses in patients with pre-cancerous cervical lesions (so-called high grade cervical intraepithelial neoplasia, CIN III) have been reported not only against HPV E6 and E7 proteins but also against the L1 capsid protein (Nimako *et al.*, 1997; Shepherd *et al.*, 1996). Primarily these responses were CD4 mediated responses against L1 de-

rived peptides of 15 amino acids in length as assessed by short-term culturing of T cells, but also CD8 mediated proliferative responses against L1 were observed. The number of responders was significantly higher in patients with HPV-16 positive biopsies compared to those with negative lesions. Generally the L1 protein is thought to be expressed solely in terminally differentiated epithelial cells (Stoler *et al.*, 1992; Taichman and LaPorta, 1987), which represents only a small portion of the infected cells within a lesion. However, these cells are the producer cells of new virus particles and eradication of these cells would prevent the further spreading of the viral infection. It has to be investigated to what extent a VLP based vaccine could help to prevent further spread of the virus in human patients by eliminating the producer cells. Our results indicate the possibility of such an approach. Additionally, fusion of the L1 and/or L2 protein to the E6 or E7 proteins generates chimeric VLPs, which are able to induce not only capsid protein-specific immune responses but also E6 and E7 protein-specific immune responses in mice (Greenstone *et al.*, 1998).

The exact mechanism by which VLPs induce a CD8 restricted immune response *in vitro* remains unclear, but the results presented in this work strongly suggest that VLPs can absorb to and gain entry into certain cells present in the human PBL population. Most likely the VLPs mimic the native virus particle and are able to escape endocytic vesicles and gain entry into the cytosol, where the capsid proteins could be degraded by proteasomes. Subsequently this leads to presentation of CTL-epitopes via the normal TAP dependent MHC class I presentation pathway. Nevertheless, it is not clear which cells of human PBL are taking up VLPs and present CTL-epitopes. It is of interest to note the very strong proliferative response to the VLPs in the cultured PBL. This could have two obvious explanations, namely a strong memory response due to prior HPV-16 infection of the donor used in these studies, or an activation of the PBL due to cross-linking of a surface receptor. There is no good method to test human patients for lifetime exposure to HPV and the presence of antibodies against L1-proteins are highly specific for the HPV type, as well as not always present. Serum of both donors used in our experiments was tested for the presence of anti-HPV16-L1 antibodies. The male donor was found negative, meanwhile the female donor was very weakly positive, which could indicate a exposure to HPV16 during her lifetime. Nevertheless a Pap-smear was found negative. Considering the very low anti-L1 antibody titer in the serum, we assume that the female donor is currently HPV16 negative, too. It has to be pointed out that the observed T cell responses could reflect a memory response to L1 or L2 protein due to previous exposure to HPV-16, at least in the case of the female donor. However, the male donor did not show any indication of a previous exposure to HPV-16 but showed identical results upon cultivation of non-adherent PBL in the presence of HPV-16 L1-VLPs or HPV16-L1L2 VLPs. This result indicates a possible primary induction of an HPV16 capsid protein-specific CTL response.

Recent reports point out a candidate receptor for papilloma virus composed of an α_6 integrin and either a β_1 or β_4 integrin (Evander *et al.*, 1997). Nevertheless, only epithelial cells, immatur thymocytes and several types of tumor cells express the $\alpha_6\beta_4$ integrin (Evander *et al.*, 1997; Müller *et al.*, 1995; Qi *et al.*, 1996; Sonnenberg *et al.*, 1990; Wadsworth *et al.*, 1992). Moreover, VLPs have been used in the past to characterize the binding of papilloma viruses to a wide range of different cell types and tissue types, indicating a more general and wider expression of the receptor than the $\alpha_6\beta_4$ integrin (Müller *et al.*, 1995; Qi *et al.*, 1996; Sonnenberg *et al.*, 1990; Wadsworth *et al.*, 1992; Volpers *et al.*, 1995). This suggests the presence of more than only one receptor. The exact nature of the receptor(s) remains unclear, but it is presumably present on at least some of human PBL capable of stimulating a primary or a secondary MHC class I restricted CTL response.

The presence of one or more strong CTL epitopes within the L1 or the L2 proteins may pose a possible complication for the use of L1L2-VLPs as carriers for tumor vaccines (chimeric VLPs). The results of our analysis with HPV16 VLPs indicate that the capsid protein(s) are recognized by the cellular immune system. On the one hand an immunodominant CTL epitope within the capsid protein(s) could hamper a strong tumor antigen-specific CTL response after vaccination with chimeric VLPs. On the other hand the CTL responses to capsid protein(s) should allow monitoring the progress of immunizations with chimeric VLPs in human clinical trials.

Materials and Methods

Production of VLPs and Capsomers

HPV-16 L1L2-virus like particles (VLPs) were produced as described earlier (Greenstone *et al.*, 1998). For production of mutated L1(152) capsomers, a PCR fragment amplifying part of the HPV 16 L1 ORF and spanning the *Bam*HI and *Eco*NI restriction sites was generated using the primers AAAGGATCCCCAAGCTACCAATGTTGCAGTAAATCC and GCCTGGGATGTTACAAACCTATAAGTATCTTC and the HPV 16 L1 genomic clone (114/k) as template. The underlined sequence indicates the codon change TGT (Cys) into AGT (Ser). The fragment was then digested with *Bam*HI and *Eco*NI and used to exchange a corresponding *Bam*HI/*Eco*NI fragment of the L1 ORF. The mutated L1 gene was inserted into the *Xba*I/*Sma*I site of pVL1392 resulting in the plasmid pVL16L1 Cys ⇒ Ser. Recombinant baculoviruses were generated by co-transfection of the plasmid pVL16L1Cys ⇒ Ser with linearized genomic baculovirus DNA (Baculo Gold, Pharmingen, San Diego, CA, USA) into Sf9 insect cells using the calcium-phosphate method. Recombinant viruses were isolated using the manufacturers recommendations and used for the infection of High-Five insect cells for production of HPV-16 L1(152) capsomers by adapting a protocol originally developed to purify HPV 16 L1 VLPs (Müller *et al.*, 1997).

Cell Mediated Lymphocytotoxicity Assay

PBL from two healthy HLA-A*0201 positive donors were obtained by leukapheresis and stored in liquid nitrogen for further use. One donor is male, the other donor is female. Both were tested for

antibodies against HPV16 L1 in the serum. The male donor was found to be negative, meanwhile the female donor, who was Pap-smear negative, was found to have a very weak positive antibody response to HPV16 L1 protein (data not shown). Cells were thawed, washed once with RPMI1640, containing 10 mM pyruvic acid, 10 mM non-essential amino acids, 100 μg/ml Kanamycin, 5% human AB-serum (Sigma, St. Louis, MO, USA), and plastic adherent cells were removed by plating 15×10^6 cells/ml in 175 cm^2 tissue culture flask for 2 hours at 37°C (Zhou *et al.*, 1991). Non-adherent cells were collected and 25×10^6 cells were incubated with 10 μg/ml HPV16-L1L2 VLPs for 1 hour at room temperature. Cells were cultured in 48-well plates (Costar, Cambridge, MA, USA) at 0.5×10^6 cells per well for 7 days at 37°C. Re-stimulation was done with 0.5×10^6 cells per well of non-adherent PBL, incubated with 10 μg/ml HPV16-L1L2 VLPs for 1 hour at room temperature. After 7 days the cells were pooled, purified over Lymphoprep (Nycomed, Oslo, Norway) and tested for specific lysis on target cells. Target cells (HLA-A*0201 positive human lymphoma cell line JY) were incubated overnight with 10 μg/ml HPV16-L1L2 VLPs, washed once with PBS and labeled with [^{51}Cr] for 1 hour at 37°C. Effector cells were incubated at different effector/target ratios in the presence of a 20-fold excess of K562-cells (to block natural killer cell activity) in triplicates for 4 hours at 37°C. 50 μl of culture supernatant was mixed with 125 μl Microscinth-40 and activity of released [^{51}Cr] was measured in a Packard TopCount Microplate Scintillation Counter (Packard Instrument Company, Meriden, CT, USA). Lysis was calculated as % specific lysis = 100 × [cpm(sample)-cpm(spontaneous release)]/[cpm(maximum release)-cpm(spontaneous release)].

Proliferation Assay

Non-adherent PBL were incubated in triplicates for 3 days with 10 μg/ml HPV-16 L1-VLPs or HPV16-L1L2 VLPs, mutated L1 (152) capsomers or no stimulus in 96-well plates (Costar, flat-bottom). ^{3}H-thymidine was added (0.4 μCi/well) and plates were incubated for 6 hours at 37°C. Cells were collected with a cell harvester on trans-plates and incorporated activity was measured in a Packard TopCount Microplate Scintillation Counter (Packard Instrument Company) following manufacturers instructions.

TNFα Assay

Adherent human Rhabdomyosarcoma RD cells [ATCC CCL-136, positive for HLA-A1, BW51 and B14 (Mazlarz *et al.*, 1986)] were grown to a continuous layer in 6-well plates in IMDM + 10% FCS and transfected with 2.5 ng of human HLA-A*0201 in pCDNA3 (Invitrogen, Carlsbad, CA, USA) using 10 μg lipofectamine (Gibco BRL, Gaithersburg, MD, USA) in OptiMem serumfree medium (Gibco BRL) according to manufacturer's instructions. Cells were harvested after 24 hours and used to stimulate 10^4 effector cells at the indicated combinations in 96-well plates in the presence of 20 U/ml IL-2. After incubation overnight at 37°C, 50 μl supernatants were tested for the presence of TNFα in a WEHI/MTT assay (Traversari *et al.*, 1992).

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Immunology

Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction

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ABSTRACT **Vaccination with synthetic peptides representing cytotoxic T lymphocyte (CTL) epitopes can lead to a protective CTL-mediated immunity against tumors or viruses. We now report that vaccination with a CTL epitope derived from the human adenovirus type 5 E1A-region ($Ad5E1A_{234-243}$), which can serve as a target for tumor-eradicating CTL, enhances rather than inhibits the growth of Ad5E1A-expressing tumors. This adverse effect of peptide vaccination was rapidly evoked, required low doses of peptide (10 μg), and was achieved by a mode of peptide delivery that induces protective T-cell-mediated immunity in other models. Ad5E1A-specific CTL activity could no longer be isolated from mice after injection of Ad5E1A–peptide, indicating that tolerization of Ad5E1A-specific CTL activity causes the enhanced tumor outgrowth. In contrast to peptide vaccination, immunization with adenovirus, expressing Ad5E1A, induced Ad5E1A-specific immunity and prevented the outgrowth of Ad5E1A-expressing tumors. These results show that immunization with synthetic peptides can lead to the elimination of anti-tumor CTL responses. These findings are important for the design of safe peptide-based vaccines against tumors, allogeneic organ transplants, and T-cell-mediated autoimmune diseases.**

Cytotoxic T lymphocytes (CTL) can play an important role in the defense against experimental and human malignancies (1, 2). CTL recognize small antigenic peptide fragments in the context of class I major histocompatibility molecules (3). These peptides are mostly generated from endogenously synthesized proteins.

Vaccination with synthetic peptides corresponding to CTL epitopes can induce protective CTL-mediated immunity in a variety of model systems (4). For instance, immunization of mice with synthetic peptides deduced from proteins of either Sendai virus or lymphocytic choriomeningitis virus (LCMV) led to the induction of protective immunity against a subsequent challenge of respectively Sendai virus or LCMV (5, 6). In tumor models, protective immunity was established by immunization with tumor-specific synthetic peptides. Immunization with a peptide derived from the human papillomavirus type 16 (HPV16) led to the protection against a lethal dose of HPV16-transformed tumor cells (7, 8), and vaccination with a peptide encompassing a CTL epitope derived from chicken ovalbumin led to the induction of protective immunity against a thymoma transfected with the cDNA of chicken ovalbumin (9). The approach of using synthetic peptide CTL epitopes for the induction of CTL responses is now being applied in human beings. Peptides encoded by the HPV16 early region 6 and 7 (E6 and 7) oncogenes that are immunogenic to human CTL have been identified (10) and are currently employed at our hospital in a phase I/II peptide vaccination study. Vaccination of healthy volunteers with the hepatitis B core antigen peptide 18–27 linked to a T-helper epitope and two palmitic acid molecules induced a hepatitis B core antigen peptide-specific CTL response cross-reactive on virus-infected cells (11).

While these results provide the basis for the development of peptide-based prophylactic and therapeutic anti-tumor vaccines, vaccination with synthetic peptides may also lead to T cell unresponsiveness (12, 13). At least three repetitive intraperitoneal (i.p.) immunizations with a relatively high dose (100 μg/mouse) of synthetic peptide deduced from the glycoprotein of LCMV induced specific T-cell tolerance, and this mode of peptide delivery prevented the induction of diabetes by infection with LCMV in a transgenic mouse model in which LCMV glycoprotein was expressed in the β islet cells of the pancreas (12). Apparently, there is a balance between induction of T-cell responses and tolerization by injection of antigenic peptides. These differences in outcome of peptide vaccination could have a strong impact on the design, delivery, and development of peptide-based anti-tumor vaccines, but will also have implications for vaccination strategies against T-cell-mediated autoimmune diseases and other harmful T-cell-mediated immune destructions. In this paper we show that a single vaccination with a low dose of a subcutaneously (s.c.) given peptide comprising a CTL-epitope derived from the human adenovirus type 5 early region 1A (Ad5E1A) oncogene promotes rather than suppresses the outgrowth of Ad5E1A-expressing tumor cells in normal immunocompetent mice. The inhibitory effects of peptide vaccination were also noted on adoptively transferred Ad5E1A-specific CTL clones and in T-cell receptor (TCR) transgenic mice. The implications of these findings for the development of peptide-based intervention protocols against T-cell-mediated autoimmune diseases, allogeneic organ transplants, viral infections, and malignancies are discussed.

MATERIALS AND METHODS

Mice. C57BL/6 (B6 Kh, H-2^b) mice were obtained from the Netherlands Cancer Institute (Amsterdam) and C57BL/6 *nu/nu* (B6 nude) were obtained from Bomholtgard (Ry, Denmark). The TCR transgenic mice express the TCR-α and -β chains derived from the H-$2D^b$-restricted, $Ad5E1A_{234-243}$-specific CTL clone 5 (14). This T cell uses rearranged Vα16 and Vβ1 TCR chains. The cDNAs encoding the complete α and β chains were inserted into an expression construct based on genomic sequences of the human CD2 gene (15), and the resulting transgenes were coninjected into blastocysts of C57BL/6 (B6 Kh) mice.

Cell Lines and Culture Conditions. Cells expressing Ad5E1A and E*Jras* (AR-cell lines) were generated by transfection with pAd5E1A (*PstI*) (16), pE*Jras* (17), and pTK-neo

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Abbreviations: Ad5E1, adenovirus type 5 early region 1; CFA, complete Freund's adjuvant; CTL, cytotoxic T lymphocyte; HPV, human papillomavirus; IFA, incomplete Freund's adjuvant; IL-2, interleukin 2; rIL-2, recombinant IL-2; LCMV, lymphocytic choriomeningitis virus; TCR, T-cell receptor.

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(14). Expression of Ad5E1A and E*Jras* gene products was confirmed both at the mRNA and protein level (data not shown). All other cell lines used were generated as described (14, 18). All cells were maintained as described elsewhere (7, 14, 18).

Peptides. Peptides were generated by solid phase strategies on a ABIMED 422 synthesizer (ABIMED, Langenfeld, Germany) as described (19). Peptides were stored dry at −70°C. The purity of the peptides was determined by analytical phase HPLC using water-acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA) and proved to be at least 85% (UV, 214 nm). The integrity of the peptides was determined by laser desorption time-of-flight mass spectrometry (TOF-MALDI) on a lasermat mass spectrometer (Finnigan–MAT, Herts, UK). About 5 pmol of the peptide in 0.5 μl water/acetonitrile containing 0.1% TFA was mixed with 0.5 μl of matrix solution [ACH; 10 mg/ml in acetonitrile/water, 60/40 (vol/vol) containing 0.1% TFA] and applied to the instrument. Calibration was performed with peptides of known molecular mass.

Peptide Immunization and Challenge with Ad5E1A + ras Transformed Tumor Cells. Peptide immunizations were performed as previously described (5–7). Peptides dissolved in 100 μl phosphate-buffered saline (PBS) were extensively mixed with 100 μl incomplete Freund's adjuvant (IFA) and 0.5% (wt/vol) of bovine serum albumin (BSA). The 200 μl mixture was s.c. injected in B6 mice. Two weeks later mice were challenged with 10^7 Ad5E1A + ras transformed tumor cells (clone AR 6) or 0.5×10^6 HPV16-transformed tumor cells (clone HPVC3) s.c. in 300 μl PBS.

Limiting Dilution. CTL clone 5 (1.5×10^7) (14) was administered intravenously in normal immunocompetent mice. Three days later 100 μg of the Ad5E1A-encoded CTL epitope $Ad5E1A_{234-243}$ (sequence, Ser-Gly-Pro-Ser-Asn-Thr-Pro-Pro-Glu-Ile) or the $Ad5E1B_{192-200}$-encoded (control) CTL epitope (sequence, Val-Asn-Ile-Arg-Asn-Ile-Cys-Cys-Tyr-Ile) (18) in PBS was mixed with IFA plus 0.5% (wt/vol) BSA and s.c. injected. Six days later spleen cells of these animals were plated out in a limiting dilution assay. Twenty-four replicate microcultures were set up per serial dilution of responder spleen cells and 5×10^3 irradiated (25 Gy) interferon-γ (2 days, 10 units/ml) Ad5E1-transformed cells in U-bottomed tissue culture plates. After 7 days of culture in the presence of 10 Cetus Units of recombinant interleukin 2 (rIL-2) the microcultures were split into three and tested in an Europium- (Eu^{3+}) release assay (7) on 10^3 Eu^{3+}-labeled syngeneic target cells in a total volume of 100 μl in the presence of 0.5 μM of peptide $Ad5E1A_{234-243}$ (14), peptide $Ad5E1B_{192-200}$ (18), or the HPV16 $E7_{49-57}$-encoded peptide Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe (7). Microcultures were scored as responding and nonresponding cultures. Responding cultures were defined as those in which the Eu^{3+} release value exceeded the mean background Eu^{3+} release plus three times the standard deviation. Limiting dilution analysis predicts that if 37% of the tested microcultures is negative, then for that given responder cell concentration there is an average of one CTL-precursor per well (20). CTL-precursor frequencies were only taken into account if the goodness of fit was ≤12.5 (Jacknife method; ref. 21).

***In Vivo* Administration of Tumor-Specific CTL Clones.** *In vivo* therapy for Ad5E1-induced tumors with tumor-specific CTL clones was performed as described (14). In short, B6 *nu/nu* mice with Ad5E1-induced tumors ranging from 40–50 mm^3 were treated with intravenous injections of B6 Ad5E1A-specific CTL clone 5 (1.5×10^7) in combination with 10^5 Cetus Units rIL-2, administered s.c. mixed with 100 μg of peptide $Ad5E1A_{234-243}$ or 100 μg of peptide $Ad5E1B_{192-200}$ as a control in IFA containing 0.5% BSA at a site distant from the tumor. The mixtures of peptide/IL-2 and IFA were prepared as described above.

RESULTS

Generation of Ad5E1A + ras Transformed Tumor Cells. Previously, we described a CTL epitope encoded by the Ad5E1A region ($Ad5E1A_{234-243}$). This CTL epitope is presented to the immune system in the context of H-$2D^b$, and CTL clones directed against this epitope are able to eradicate large established Ad5E1-induced tumors in B6 nude mice (14, 22). This indicates that the Ad5E1A-encoded epitope is able to elicit CTL capable of mediating tumor regression of Ad5E1A-expressing tumors *in vivo*. To test whether vaccination with this CTL epitope is also able to induce protective immunity against Ad5E1A-expressing tumor cells in immunocompetent mice, we generated tumor cells transformed by the Ad5E1A region and an activated *ras* oncogene. The Ad5E1A + ras transformed cells are recognized by Ad5E1A-specific CTL clone 5, showing that these tumor cells present the $Ad5E1A_{234-243}$-encoded CTL epitope (data not shown). Moreover, Ad5E1A + ras transformed tumor cells, in contrast to Ad5E1-transformed cells, are tumorigenic in immunocompetent mice, so that they can be used to study the effects of vaccination with peptide $Ad5E1A_{234-243}$ on the induction of protective CTL mediated immunity against tumor outgrowth *in vivo*. A dose of 10^7 tumor cells injected s.c. causes growth of small tumors in 80–100% of the mice. By day 40, ≈10–30% of the animals die because of a progressively growing tumor. The other animals, which have developed a tumor, still carry it or have eradicated the tumor (5–10% of the animals) (data not shown), indicating that Ad5E1A + ras transformed tumor cells are weakly immunogenic in immunocompetent mice.

Immunization with the Ad5E1A-Encoded CTL Epitope Leads to an Enhanced Outgrowth of Ad5E1A + ras Expressing Tumor Cells. Immunocompetent B6 mice were immunized once s.c. with peptide $Ad5E1A_{234-243}$ in IFA, a vaccination protocol that has been successfully used to induce protective immunity in several other models (5–7). Two weeks later the mice were challenged with Ad5E1A + ras transformed tumor cells. Control mice immunized with irradiated tumor cells were protected against the outgrowth of Ad5E1A + ras transformed tumor cells, indicating that protective immunity can be established against these tumor cells (Fig. 1). Unexpectedly, mice immunized with the Ad5E1A peptide were not protected against the outgrowth of these tumor cells. Instead, the tumors in these animals grew faster than the tumors in animals that were injected with IFA only, or with the H-$2D^b$-binding control peptide HPV16 $E7_{49-57}$ in IFA (Fig. 1*A*). All animals vaccinated with the Ad5E1A peptide died within 40 days after tumor challenge, whereas at that time almost all animals in the control groups were still alive (Fig. 1). These results indicate that immunization with peptide $Ad5E1A_{234-243}$ leads to the inability of immunized mice to control the outgrowth of Ad5E1A-expressing tumors. This effect is induced very rapidly, since injection of 10 μg of the Ad5E1A peptide at the left flank and a tumor cell challenge on the right flank of the animal on the same day resulted in the enhancement of tumor outgrowth (Fig. 1*C*).

To define the minimum amount of peptide required to observe the enhancement of tumor outgrowth, we titrated the injected dose of peptide (Fig. 2). Mice injected with 10 μg of the Ad5E1A peptide develop rapidly growing tumors earlier and died sooner than animals injected with lower doses of peptide or an H-$2D^b$-binding control peptide ($P = 0.004$; log-rank test) (Fig. 2). Protective immunity against the outgrowth of Ad5E1A + ras cells could not be induced at any concentration of peptide (ranging from 10 μg to 1 ng peptide per mouse), since the tumor-take in mice receiving the Ad5E1A peptide was the same as the tumor-take in mice receiving the Ad5E1B control peptide (data not shown).

Administration of the Ad5E1A peptide, and not of other peptides (Figs. 1 and 2), leads to the enhanced outgrowth of

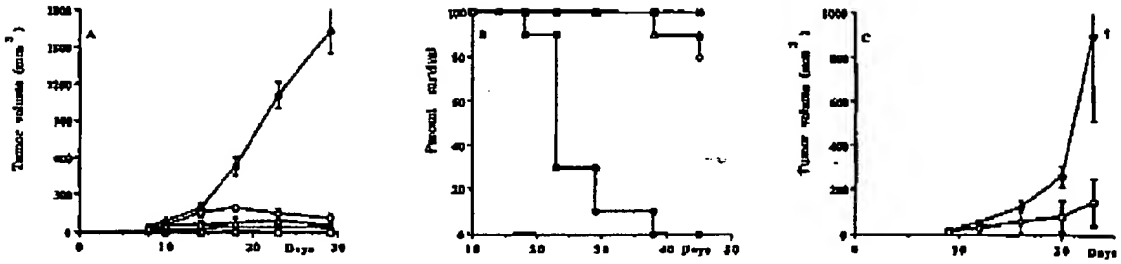

FIG. 1. Vaccination with the Ad5E1A peptide results rapidly in the inability to control the outgrowth of Ad5E1A + ras transformed tumor cells. Mice were immunized s.c. with irradiated Ad5E1A + ras cells in PBS (□), left untreated (—), immunized with 50 μg of peptide $Ad5E1A_{234-243}$ in IFA (●), with 50 μg control peptide HPV16 $E7_{49-57}$ in IFA (○), or with IFA only (△). Two weeks later the mice were challenged with live Ad5E1A + ras cells on the other flank. Tumors in mice immunized with Ad5E1A peptide grow more rapidly compared with tumors in animals treated with control peptide ($P < 0.0001$; one-sided Student's *t* test). Mean tumor volumes ± SEM ($n = 10$) are shown in mm^3 (*A*). Mice immunized with Ad5E1A peptide die sooner because of a progressively growing Ad5E1A + ras tumor compared with mice injected with control peptide ($P = 0.0006$; log-rank test) (*B*). The enhanced outgrowth of Ad5E1A + ras transformed tumor cells is induced rapidly after peptide vaccination (*C*). Mice were immunized s.c. on day zero with 10 μg of the Ad5E1A peptide in IFA or with 10 μg of the HPV16 E7-encoded control peptide in IFA and on the same day, on the other flank, injected with live Ad5E1A + ras transformed tumor cells. Mice receiving Ad5E1A peptide develop bigger tumors than mice injected with control peptide. Mean tumor volumes ± SEM ($n = 5$) are shown in mm^3.

Ad5E1A-expressing tumors. This shows that these tumors do not grow more rapidly when control peptides are injected, but only when the Ad5E1A peptide is given. Thus, this effect is peptide-specific. To show that immunization with the Ad5E1A peptide leads to the enhanced outgrowth of Ad5E1A-expressing tumors, and not of other tumor types, we immunized B6 mice with the HPV16 $E7_{49-57}$-derived CTL epitope mixed with the $Ad5E1A_{234-243}$ peptide. Mice were challenged with HPV16-transformed tumor cells. Immunization with peptide HPV16 $E7_{49-57}$ induces protective CTL-mediated immunity against HPV16-induced tumors (7). Mice immunized with the mixture of Ad5E1A and HPV16 E7 peptides in IFA are equally well protected against a subsequent challenge of HPV16-transformed tumor cells as mice immunized with HPV16 E7 peptide in IFA only (Fig. 3). Moreover, tumor growth rates of HPV16-transformed tumor cells in mice immunized with only $Ad5E1A_{234-243}$ was the same as the tumor growth rate in control mice (data not shown). These data indicate that injection of peptide $Ad5E1A_{234-243}$ does not result in a general inability of the mice to reject tumors. Taken together, these observations suggest that immunization with the Ad5E1A-encoded CTL epitope leads to a specific functional deletion of Ad5E1A-specific immunity, resulting in an inability to reject Ad5E1A-expressing tumors.

Ad5E1A-Specific Activity Cannot Be Detected After Administration of the Ad5E1A Peptide. Immunization with the Ad5E1A peptide leads to an enhanced outgrowth of Ad5E1A-expressing tumors. This suggests that Ad5E1A-specific CTL are tolerized by administration of the Ad5E1A-encoded CTL epitope. To study the effect of administration of the Ad5E1A peptide on Ad5E1A-specific CTL activity, Ad5E1A-specific CTL clone 5 was adoptively transferred into naive immunocompetent animals. Three days later peptide $Ad5E1A_{234-243}$, or an $Ad5E1B_{192-200}$-encoded control peptide (17) were administered. The presence of CTL clone 5 in the spleen of these animals was functionally tested 6 days later. CTL clone 5 could only be recovered from animals that received CTL clone 5 and the Ad5E1B control peptide, but not from animals that received CTL clone 5 and the Ad5E1A peptide (Table 1). Administration of the Ad5E1A peptide had no effect on the presence of adoptively transferred Ad5E1B-specific CTL clones (data not shown). To study whether the Ad5E1A peptide had also an effect on naive Ad5E1A-specific CTL, B6 mice expressing a transgenic TCR specific for the Ad5E1A peptide were injected with the Ad5E1A peptide. In contrast to splenocytes from normal B6 mice, splenocytes from naive TCR-transgenic mice, when restimulated *in vitro* with Ad5E1A peptide-loaded lipopolysaccharide blasts, display strong cytolytic activity against Ad5E1-transformed cells. After injection of the Ad5E1A peptide, but not of an irrelevant control peptide, the spleen cell cultures derived from Ad5E1A-specific TCR-transgenic animals are no longer able to lyse Ad5E1-transformed tumor cells (Fig. 4). Taken together, the com-

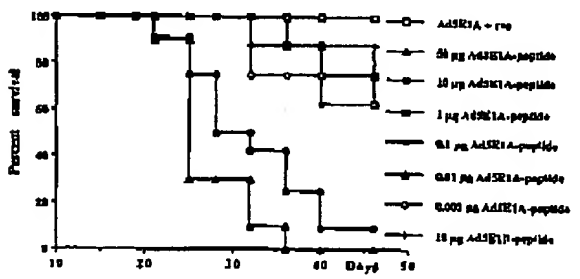

FIG. 2. Tumor growth of Ad5E1A + ras cells after titration of the $Ad5E1A_{234-243}$ peptide. Mice were immunized s.c. with irradiated Ad5E1A + ras cells, with 10 μg of the $Ad5E1B_{192-200}$-encoded control peptide (18) in IFA, or with 50 to 0.001 μg of the Ad5E1A peptide in IFA. Two weeks later mice were challenged with live Ad5E1A + ras cells on the other flank. Shown is the percentage of surviving animals.

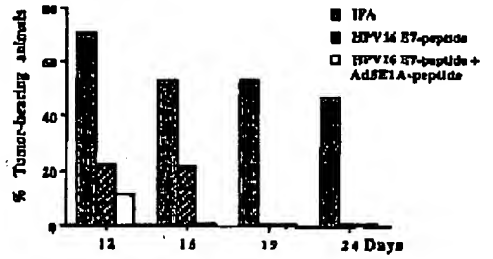

FIG. 3. Effect of immunization with the $Ad5E1A_{234-243}$ peptide is tumor-specific. Mice were immunized with 10 μg of the HPV16 $E7_{49-57}$ peptide in IFA, with a mixture of 10 μg HPV16 E7 peptide and 10 μg Ad5E1A peptide in IFA or with IFA only. Two weeks later mice were challenged with HPV16-transformed tumor cells. The HPV16 $E7_{49-57}$ peptide induces protective immunity against a challenge with HPV16 E7-transformed tumor cells (7). Mice immunized with the peptide mixture are also protected ($P = 0.03$, log-rank test). Shown is the percentage of tumor-bearing mice on several days after tumor challenge.

Table 1. Adoptively transferred Ad5E1A-specific CTL clone 5 is functionally deleted by the Ad5E1A peptide in vivo

	RMA + $E7_{49-57}$	RMA + $E1B_{192-200}$	RMA + $E1A_{234-243}$
Exp. 1			
Clone 5 + $E1B_{192-200}$	1 ± 1	2 ± 1	80 ± 10
Clone 5 + $E1A_{234-243}$	2 ± 1	4 ± 2	4 ± 2
Exp. 2			
Clone 5 + $E1B_{192-200}$	2 ± 1	8 ± 1	174 ± 21
Clone 5 + $E1A_{234-243}$	14 ± 2	17 ± 2	13 ± 2

Ad5E1A-specific CTL clone 5 (1.5×10^7) was adoptively transferred by intravenous injection to naive immuno-competent B6 mice at day 0. At day 3 these animals received s.c. the Ad5E1A peptide or the Ad5E1B peptide in IFA. At day 9 the spleen cells were taken and put in limiting dilution. At day 16 the contents of the wells were tested on syngeneic RMA cells loaded with 0.5 μM of the HPV16 $E7_{49-57}$-encoded control peptide, the $Ad5E1B_{192-200}$-encoded control peptide, or the $Ad5E1A_{234-243}$-encoded peptide. Values in table show minimum estimates of CTL ± SD per 10^6 spleen cells.

bined data on tumor outgrowth and Ad5E1A-specific CTL activity after peptide immunization indicate that both naive and preactivated Ad5E1A-specific CTL are functionally deleted by administration of the $Ad5E1A_{234-243}$-encoded CTL epitope.

The Ad5E1A Peptide Rapidly Diffuses Through the Body After s.c. Administration in IFA. The data described above indicate that intravenously injected Ad5E1A-specific CTL clone 5 is functionally deleted *in vivo* by s.c. administration of the Ad5E1A-encoded CTL epitope. CTL clone 5 is able to eradicate established Ad5E1-induced tumor in nude mice (14). To test if administration of the Ad5E1A-peptide leads to the inability of CTL clone 5 to eradicate established Ad5E1-induced tumors *in vivo*, we treated Ad5E1 tumor-bearing *nude* mice by intravenous injection of CTL clone 5. At the same time the Ad5E1A peptide, or as a control, the $Ad5E1B_{192-200}$-encoded peptide, was given s.c. together with rIL-2 in IFA. Unexpectedly, the animals that received CTL clone 5 and the Ad5E1A peptide died within 16 h (Table 2), whereas animals that received only the Ad5E1A peptide or CTL clone 5 together with the Ad5E1B peptide survived (Table 2). Mice that received an Ad5E1B-specific CTL clone in combination with the Ad5E1A peptide survived and eradicated the Ad5E1-induced tumors (data not shown). Similar findings were obtained in immunocompetent animals that received CTL clone 5 at the same time as the Ad5E1A peptide, together with rIL-2. Autopsy revealed that animals receiving the combination of

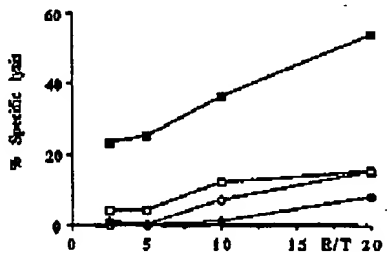

FIG. 4. Injection of the Ad5E1A peptide deletes naive Ad5E1A-specific CTL activity. Naive B6 mice, expressing a transgenic TCR specific for the Ad5E1A peptide, were injected with 10 μg of the $Ad5E1A_{234-243}$ peptide (open symbols) or 10 μg of the HPV16 $E7_{49-57}$ peptide (solid symbols) s.c. in IFA. Six days later 5×10^6 spleen cells of these animals were restimulated with 25% Ad5E1A peptide-loaded lipopolysaccharide blasts in a 24-well plate. After 5 days of culture the bulk CTL cultures were tested on untransformed B6 MEC (●, ○) or Ad5E1-transformed tumor cells (■, □). Shown is the percentage of specific lysis at different effector-to-target cell ratios.

Table 2. Combination therapy with adoptively transferred CTL clone 5 and Ad5E1A peptide leads to the death of mice.

Treatment	$Ad5E1A_{234-243}$, %	$Ad5E1B_{192-200}$, %
—	0	0
CTL clone 5, i.v.	100	0

Ad5E1-tumor-bearing *nude* mice (5 animals per group) were treated with 1.5×10^7 Ad5E1A-specific CTL clone 5 by intravenous (i.v.) injection. At the same day these mice received 10^3 Cetus units rIL-2 mixed with 100 μg peptide in IFA. Sixteen hours later all animals receiving Ad5E1A-specific CTL clone 5 and Ad5E1A peptide were dead. In all animals receiving CTL clone 5 and the Ad5E1B-encoded control peptide, tumors were eradicated. Values in table show percentage of dead mice.

CTL clone 5 and Ad5E1A peptide had severely congested lungs (data not shown). Apparently, the adoptively transferred Ad5E1A-specific CTL that are trapped in the capillary bed of the lungs become activated by injection of the Ad5E1A peptide, leading to the observed lung pathology. The mechanisms involved in induction of this lung pathology falls beyond the scope of this study and are now under further investigation. Nonetheless, these results show that s.c. administrated $Ad5E1A_{234-243}$ peptide in IFA rapidly diffuses throughout the body where it is recognized by Ad5E1A-specific CTL.

Protective Immunity Induced by Vaccination with Adenovirus. Immunization with the Ad5E1A peptide leads to Ad5E1A-specific CTL tolerance and severely affects the ability of B6 mice to cope with Ad5E1A-expressing tumors. Immunization with irradiated tumor cells, however, induces protective immunity against these tumor cells, showing the feasibility to induce protective immunity against Ad5E1A + ras transformed cells. Vaccination with irradiated tumor cells will, however, be difficult to execute on a large scale in a clinical setting. Recombinant adenoviruses might serve as efficient vaccine vehicles for the induction of protective anti-tumor immunity. Recombinant adenoviruses are currently being tested for their ability to deliver genes to a spectrum of nondividing cells *in vivo* for the treatment of genetic diseases. The use of recombinant adenoviruses is associated with transient gene expression due to a T-cell-mediated immune response against vector-derived proteins (23–25). This indicates that recombinant adenoviruses might also be used to induce protective immunity against tumors, when they contain DNA encoding for tumor antigens or tumor-derived T-cell epitopes. To test if adenoviruses can indeed be used to establish protective immunity against tumors, we immunized B6 mice with an adenovirus type 5 variant (*ts*149). Ad5*ts*149 is largely replication deficient, but expresses, upon infection, Ad5E1A-encoded protein (26). Mice immunized with Ad5*ts*149 showed high CTL reactivity against the Ad5E1A-encoded CTL epitope (Fig. 5 *A* and *B*) and are protected against a subsequent challenge with Ad5E1A + ras cells (Fig. 5*C*). These results show the possibility to use recombinant adenoviruses expressing tumor antigens for the induction of tumor-specific protective immunity.

DISCUSSION

In the present report we show that a single s.c. immunizaton with 10 μg of the $Ad5E1A_{234-243}$-encoded CTL epitope rather than inducing protective immunity against a challenge of Ad5E1A-expressing tumor cells instead causes faster tumor outgrowth. This effect is peptide-specific and is rapidly induced. Ad5E1A-specific CTL activity can no longer be detected after injection of the Ad5E1A peptide from animals that express an Ad5E1A-specific transgenic TCR or that have received Ad5E1A-specific CTL clone 5 by adoptive transfer. Although the latter observation might be a consequence of an altered migratory pattern of the Ad5E1A-specific CTL clone,

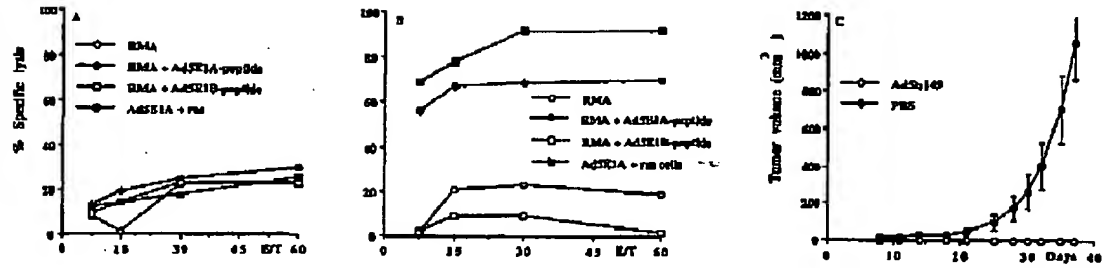

FIG. 5. Vaccination with a mutant adenovirus type 5, expressing Ad5E1A, induces Ad5E1A-specific CTL activity and protects against a subsequent challenge with Ad5E1A + ras cells. B6 mice were immunized (i.p.) with PBS + 0.5% BSA (*A* and *C*) or 2×10^9 plaque forming units of Ad5*wt*149 in PBS + 0.5% BSA (*B* and *C*). Two weeks after immunization, the spleen cells of these mice were restimulated *in vitro* with 10% Ad5E1A + ras cells (*A* and *B*), or the mice were challenged (s.c.) with Ad5E1A + ras cells (*C*). The lytic activity of bulk CTL were tested after 5 days of culture against Ad5E1A + ras cells, or syngeneic RMA cells loaded with 0.5 μM of the $Ad5E1B_{193-209}$-encoded control peptide or the $Ad5E1A_{234-243}$ peptide. Shown are the percentages of specific lysis at different effector-to-target cell ratios (*A* and *B*). The mean tumor volumes ± SEM ($n = 7$) of mice challenged with tumor cells are shown in mm^3 in *C*.

a more likely explanation is functional deletion of Ad5E1A-specific CTL. The Ad5E1A peptide rapidly diffuses throughout the body after s.c. injection in IFA and enhanced tumor outgrowth is also observed when the tumor challenge is given near the peptide–IFA depot in the same lymph nodes draining area (data not shown), indicating that the Ad5E1A-specific CTL are not massively attracted to the peptide–IFA depot.

Unlike mice immunized with adeno-derived peptides, mice immunized with live adenovirus or with irradiated tumor cells are protected against a subsequent challenge with Ad5E1A-expressing tumor cells. Great effort is currently directed toward the development of successful human gene therapies employing recombinant adenoviruses for gene transfer. One obstacle in obtaining stable expression of the delivered gene in the target cells using this method is a vector directed, T-cell-mediated immune response (23–25). By introducing tumor antigens or (several) tumor-encoded T-cell epitopes in these adenovectors it is possible to induce protective T-cell-mediated anti-tumor immunity as demonstrated here for the Ad5E1A epitope.

Peptide-based vaccination has been proven to induce T-cell-mediated protective immunity in several viral and tumor model systems (5–7, 9, 27). At the same time peptides have been found capable of T-cell tolerance induction (12, 13, 28–31). Repetitive and systemic (3 × i.p.) injections of high doses of peptide derived from LCMV-induced tolerance of LCMV-specific CTL. Local s.c. injections of this peptide protected mice against LCMV infection (13). Likewise, Kearney *et al.* (31) have shown in an adoptive transfer system that TCR-transgenic T cells are tolerized by systemic intravenous or i.p. injections of the specific peptide. In contrast, the same T cells are stimulated after s.c. injection of the peptide emulsified in complete Freund's adjuvants. They hypothesize that complete Freund's adjuvant not only functions as a depot that mediates a relatively slow release of peptide for a prolonged time period, but would also activate the local antigen presenting cells because it contains Mycobacterial components. We used as an adjuvant IFA. IFA functions as a noninflammatory vehicle because it lacks bacterial components. Nonetheless, s.c administration of the Ad5E1A peptide in complete Freund's adjuvant induces, in the Ad5E1A-tumor model, the same effects as injection of the peptide in IFA. The rapid, peptide-specific effect on the ability of B6 mice to control the outgrowth of Ad5E1A-expressing tumors is, like immunization in IFA, associated with severe lung congestion when given on the same day as an adoptively transferred Ad5E1A-specific CTL clone (data not shown). Induction of T-cell tolerance has also been described in other TCR transgenic mouse models. Continuous exposure to a high dose (100–150 μg) of peptide 366–374 deduced from the nucleoprotein of influenza virus of TCR transgenic mice expressing a TCR with a specificity for this peptide resulted in a depletion of most peripheral $CD8^+$ T cells bearing the transgenic TCR (28). This depletion appeared to be the result of thymic clonal elimination as well as peripheral loss of reactive T cells. Peripheral T cells in mice transgenic for TCR reactive with a simian virus 40-encoded peptide or a pigeon cytochrome *c* peptide are depleted *in vivo* by repeated injection of high doses (100 μg peptide injected i.p., three times) of the specific peptides (29, 30). In all these models, as opposed to the models where T-cell responses are induced, repetitive and systemic injections of relatively high doses of antigenic peptides are given. We now describe that a single s.c. injection of a much lower amount of peptide in IFA can induce T-cell unresponsiveness, leading to an enhanced outgrowth of tumors in normal immunocompetent animals.

The reasons why one peptide induces protective T-cell-mediated immunity and another T-cell tolerance when administered at comparable concentrations via the same vaccination scheme are intriguing. A possible explanation is that peptides administered s.c. in IFA eliciting protective immune responses, such as the HPV16 $E7_{49-57}$-encoded peptide are retained locally, forming a gradient of antigen. The Ad5E1A peptide diffuses rapidly throughout the body, instigating downregulation of the Ad5E1A-specific CTL response. Systemic distribution might lead to massive activation of peptide-specific CTL, that, especially if associated with inappropriate costimulation, may result in the clonal exhausting of these CTL. By analogy, it has been reported in an LCMV model that virus spread and CTL induction/exhaustion are closely linked (32). Infection with LCMV–DOCILE, a virus isolate that replicates rapidly and widely when injected in mice, induced LCMV-specific CTL tolerance, whereas infection with LCMV–WE, an isolate that replicates more slowly, induced long-lasting LCMV-specific CTL memory. In this respect it is remarkable to note that administration of the HPV16 $E7_{49-57}$ peptide on the same day as intravenous injection of a HPV16 $E7_{49-57}$-specific CTL clone does not lead to severe lung congestion (R.E.M.T., M.C.W. Feltkamp, M.P.M. Vierboom, C.J.M.M., and W.M.K., unpublished data), suggesting that the HPV16 E7-peptide is retained locally.

The observation that functional T-cell-specific tolerance can be induced by low doses of peptide administered locally is not only important for the development of safe peptide-based anti-tumor and anti-virus vaccines, but may also have implications for the design of therapeutic protocols for CTL-mediated autoimmune diseases and harmful T-cell responses against allogeneic organ transplants. Modification of $CD4^+$ T-cell-mediated autoimmune diseases and immunopathologies by peptides has been previously shown in a murine model of

experimental autoimmune encephalomyelitis and in a murine model of T-cell recognition of house dust mite allergens (33, 34). Similarly, it has been shown that oral administration of major histocompatibility complex-derived allopeptides can induce donor-specific T-cell tolerance (35, 36). By specifically tolerizing harmful CTL through injections with low doses of peptides it might be possible to prevent or temper CTL-dependent diseases.

In conclusion, in contrast to earlier reports describing induction of T-cell-mediated protection against a subsequent challenge of virus or tumor cells following a single s.c. injection of synthetic peptide in adjuvant, we now describe that such vaccination schemes can also lead to a functional deletion of tumor-specific CTL. Epitope delivery by vaccination with infectious adenovirus type 5 was associated with protection and CTL memory rather than tolerance and tumor outgrowth. Our results indicate that peptide-based vaccines must be employed with caution in a human setting, because immunization with tumor specific peptides might lead to a diminished rather than a protective immune response. However, in the Ad5E1A-tumor system only a single viral antigen is expressed. In naturally occurring tumors there may be a larger number of relevant T-cell epitopes and tolerance to a single epitope may not have the same dramatic consequences as observed in the Ad5E1A-tumor system. Nonetheless, candidate peptides for human anti-virus and anti-cancer vaccines should be tested for their immunizing or tolerizing properties *in vivo* in vaccination experiments in human leucocyte antigen-transgenic mice (10). In addition, investigation of immunizing and tolerogenic modes of peptide delivery deserves detailed attention.

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